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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:
Shuyuan Zhang, Capucine Thwin, Zheng Wu, Toohyon Cho, Shawn Gallagher

Serial No.: 09/203,078

Filed: December 1, 1998

For: METHOD FOR THE PRODUCTION AND PURIFICATION OF ADENOVIRAL VECTORS

Group Art Unit: 1645

Examiner: Shanon A Foley

Atty. Dkt. No.: INRP:081

DECLARATION OF SHUYUAN ZHANG UNDER 37 C.F.R. §1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

I, Shuyuan Zhang, do declare that:

1. I am a citizen of China residing in the United States at 6015 Briar Hills, Sugarland, Texas, 77479. I am an employee of Introgen Therapeutics, Inc. ("Introgen").

2. I have been employed by Introgen for 6 years and currently hold the position of Associate Director of Production and Process Development at Introgen Therapeutics in Houston,

Texas. I have a Ph.D. in Biochemical Engineering from University of Surrey, England. I have done extensive research and development in the production and purification of retroviral, adenoviral and AAV and non-viral vectors for gene therapy applications. My *curriculum vitae* is attached as Exhibit 1.

3. I am familiar with the level of skill of scientists working in the field of gene therapy as of the October 1992 priority date of the referenced application.

4. I understand that a publication by Leu *et al.*, U.S. Patent 6,194,210, has been cited by the Examiner. I am told that this reference is used by the Examiner to dispute the patentability of claims 1-3, 8-25 and 29 as obvious. For the reasons set forth below, it is my opinion that Leu *et al.* would not by itself or in combination with other references render the present invention obvious. I am told that the Leu *et al.* disclosure is directed exclusively to Hepatitis A virus, a RNA virus and includes no mention whatsoever of adenovirus, a DNA virus that is entirely distinct from the foregoing. Furthermore, I am told that the working examples of Leu *et al.* are directed exclusively to Hepatitis A virus. In light of the facts set forth below, it is evident that one skilled in the art would not look to Leu *et al.* to solve problems relating to propagation of an adenovirus culture in that adenovirus is totally unrelated to Hepatitis A virus and each has numerous distinct properties.

5. Adenoviruses contain double-stranded DNA approximately 36 kb in length. They are covered by a capsid 70-100 nm in diameter. This differs from Hepatitis A viruses in that

Hepatitis A viruses contain positive single-stranded RNA of approximately 7.2–8.4 kb in length. Hepatitis A viruses are comprised of a small, 27-32 nm, protein capsid. The capsid is composed of 60 protein subunits, each consisting of four polypeptides VP1-VP4. The RNA strand is covalently bonded to a noncaspid viral protein (VPg) at its 5' end and to a polyadenylated tail at its 3' end.

6. Mature adenovirus capsid is composed of 252 protein subunits of which 240 are hexons and 12 are pentons. A fiber protein projects from the base of the pentons. The adenovirus core is composed of the linear double stranded DNA and four virus coded core proteins that specifically bind to the DNA. Adenoviruses encode a DNA polymerase but depend on the host cells for many of the other functions involved in synthesis of DNA. DNA replication is complex and distinct from that of other viruses; it involves covalently bound proteins at the ends of the DNA and circularization. Transcription is also complex, involving early and late genes distributed randomly along both strands of the DNA. Replication and assembly occur in the nucleus. After adenoviral replication, the adenovirus is assembled inside the host cell's nucleus resulting in the optimal stability for this virus being at a pH between 7.0-8.5. Hepatitis A viruses on the other hand, are heat- and acid-stable, and relatively detergent resistant. Stability of Hepatitis A viruses is best achieved at a pH of 3.0 or lower. These features of Hepatitis A virus are not shared by adenovirus, and would strongly suggest that purification methods useful for Hepatitis A viruses would not necessarily be relevant to adenovirus.

7. In contrast to adenoviruses, Hepatitis A viruses undergo replication and assembly in the cytoplasm. Their RNA acts as a messenger to synthesize viral macromolecules. Viral RNA replicates in complexes associated with cytoplasmic membranes via two distinct, partially double-stranded RNAs - the "replicative intermediates." One complex uses the sense RNA strand, and the other uses the antisense RNA strand as a template. RNA replication involves the synthesis of a complementary RNA which serves as a template for genome RNA synthesis. Genome RNA also serves as mRNA, being translated into a polyprotein that is cleaved into all the viral proteins including those proteins that serve as enzymes for specific cleavage.

8. Another difference between adenoviruses and Hepatitis A viruses is that the negative DNA strand of the double-stranded DNA of adenoviruses is directly transcribed into viral mRNA, whereas the positive single-stranded RNA of Hepatitis A viruses is copied into negative RNA that is transcribed into viral mRNA.

9. Adenoviruses and Hepatitis A viruses are further distinct in their routes and manner of infection of a cell. Infection of Hepatitis A virus occurs at a specific time in growth from that of adenovirus. Hepatitis A virus infection is nonlytic and usually persists indefinitely. The initial stage of infectivity of Hepatitis A virus occurs during the lag period due to residual inoculum virus which fails to be uncoated. During this stage, viral RNA is released and synthesis of protein and RNA occurs. This is followed by an exponential period at which the concentration of infectivity doubles and leads to a plateau period. Upon infection RNA synthesis decreases in the host cell and subsequently

synthesis of viral RNA in the cytoplasm occurs. Thus, while inhibiting cellular RNA synthesis in the nucleus, viral RNA synthesis in the cytoplasm occurs. This is followed by the inhibition of cellular protein synthesis and rapid synthesis of viral proteins. Following a decrease of viral protein synthesis, leakage of intracellular components occurs leading to cell death.

10. Adenovirus infection is lytic and occurs at high multiplicities. The adenovirus replicative cycle is divided into early and late phases with the late phase beginning at the onset of viral DNA replication. Host cell DNA and protein synthesis are inhibited in cells infected with most adenoviruses as viral DNA synthesis begins. Infection involves the fiber of the virus attaching to a specific receptor on the cell membrane. A decrease in pH alters the surface of the virion resulting in rupture of the endocytic vesicle which releases the virion into the cytoplasm. Adenoviruses, specifically human adenoviruses, remain cell-associated after the production of the new virus is completed. This virus-to-cell association makes it possible for concentration of large quantities of viruses.

11. It is known in the art that both Herpesvirus and Paramyxovirus consist of an envelope with surface projections whereas adenovirus does not have an envelope. Thus, these viruses as compared to adenovirus require an isotonic osmolarity in order to achieve stability and prevent damage to their envelope membrane, whereas adenoviral stability may be achieved at relatively hypertonic osmolarity, as it has no envelope. The replication cycle of Herpesvirus and Paramyxovirus is also distinct from that of adenovirus and involves proteins of the respective envelopes. Following entry into the

host cell these viruses require specific enzymes, thymidine kinase and RNA-dependent RNA polymerase respectively, for transcription. Therefore, the viruses used and mentioned in Leu *et al.* have structural and biological properties distinct from that of adenovirus, and in addition each has a specific time of infectivity from that of adenovirus.

12. The preceding descriptions and comparisons of adenovirus to Hepatitis A viruses, Herpesviruses and Paramyxoviruses provide substantial evidence that, due to the numerous dissimilarities, a teaching relating to one viral type would not necessarily be applicable to the other. Thus, there is no *a priori* expectation that propagation of Hepatitis A viruses or Herpesviruses or Paramyxoviruses would provide appropriate means for adenovirus preparations. In light of these facts, it is my conclusion that the Leu *et al.* patent does not render the above invention obvious.

13. Further, I understand that a publication by Perrin *et al.*, has been cited by the Examiner. I am told that this reference is used by the Examiner to dispute the patentability of claim 4 as obvious. For the reasons set forth below, it is my opinion that Perrin *et al.* would not by itself or in combination with other references render the present invention obvious. I am told that the Perrin *et al.* disclosure is directed exclusively to rabies virus, a RNA virus and includes no mention whatsoever of adenovirus, a DNA virus that is entirely distinct from the foregoing. Furthermore, I am told that the working examples of Perrin *et al.* are directed exclusively to rabies virus. In light of the facts set forth below, it is evident that one skilled in the art would not look to Perrin *et al.* to solve problems

relating to propagation of an adenovirus culture in that adenovirus is totally unrelated to rabies virus and each has numerous distinct properties.

14. Rabies virus and adenovirus are each quite distinct in their structure and biological properties. The rabies virus is an enveloped "budding" RNA-based rhabdovirus whereas adenovirus is a DNA capsid based non-enveloped virus of an entirely different viral family – these viruses infect and grow differently and replicate differently. Thus, there is no *a priori* expectation that propagation of rabies viruses would provide appropriate means for adenovirus preparations. In light of these facts, it is my conclusion that the Perrin *et al.* journal article does not render the above invention obvious.

15. There are a number of scientific publications that support the foregoing conclusion. I would direct the examiner to "Fields Virology" (B. N. Fields, Editor, Vol. 2, 3rd Ed., Lippincott Raven Publishers, 1996; Vol. 1, 4th Ed., Lippincott Williams Wilkins Publishers, 2001), and "The Adenoviruses" (H. S. Ginsberg, Editor, Plenum Press, 1984). I have attached relevant excerpts from these texts as Exhibit 2 to this declaration.

16. I hereby declare that all statements made herein of my knowledge are true, and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the referenced patent application or any patent issued thereon.

02 Mar 04

Date

Shuyuan Zhang, Ph.D

CURRICULUM VITA

Shuyuan Zhang, PhD

Working address

Introgen Therapuetics, Inc.
2250 Holcombe Blvd
Houston, TX77030
Tel 713-6104091, Fax 713-797-9913
E-mail: s.zhang@introgen.com

Home address

6015 Briar Hill Court
Sugarland, TX 77479
Tel 281-980-9304

EDUCATION

University of Surrey, UK, 1990-1993

PhD in Animal Cell Technology, February 1993
Thesis: Bubble Oxygenation of Both Low and High Density Animal Cell Cultures for Monoclonal Antibody Production

East China University of Science and Technology, Shanghai, China, 1984-1987

M.Sc. in Biochemical Engineering, February 1987
Thesis: Mixing in Filamentous Fermentation Process

Tianjin University, Tianjin, China, 1980-1984

B.Sc. in Biochemistry, September 1984

WORK EXPERIENCE

Associate Director, Production and Process Development, January, 2002- present
Introgen Therapuetics, Inc. Houston, TX
Directing Process Development activities for next generation adenoviral vector production process
Directing overall Process Validation efforts for CMC filing
BLA filing activities

Associate Director, Process Development, June 1990-January, 2002

Targeted Genetics Corp, Sharon Hill, PA

Directing the Process Development Department activities for both adenoviral and adeno-associated viral vector product process development and production.

Process Development involves cell culture, scale up to large bioreactors, downstream processing, tangential flow filtration for concentration and diafiltration, and chromatographic purification development and use of variety of analytical characterization methods. Production of the viral vectors using the developed processes under GLP and GMP conditions.

In charge of technology transfer and implementation in a Contract Manufacturing Organization for clinical production of viral vectors under GMP regulation

Manager, Process Development, October 1995-June 1999
Introgen Therapeutics, Inc., Houston, TX

Managed process development for upstream cell culture, downstream processing and chromatographic purification/characterization and formulation activities for retroviral and adenoviral vector products. Production of the vectors using developed processes

Actively involved in the collaboration with a large pharmaceutical company on adenoviral vector product development under GMP regulations

Key personnel in making production, purification and formulation process decisions

Actively participated in GMP production of adenoviral vector

Research Scientist, Process Scale Up, February 1993- October 1995
Genetic Therapy, Inc, Gaithersburg, MD

Responsible for process development, scale up and production of retroviral vectors from a variety of producer cells

Successfully developed large-scale retroviral vector production and purification process for clinical applications. The development resulted in the issuance of two US patent.

Large-scale production of clinical grade retroviral vectors

Research Associate, February 1987- January 1990
East China University of Science and Technology, Shanghai, China

PROCESS DEVELOPMENT AND MANUFACTURING EXPERIENCE

1. Successful development of a production process for retroviral vector production including upstream bioreactor design, downstream tangential flow filtration concentration and chromatographic purification and lyophilization.
2. Successful development of a microcarrier culture based production process for large-scale adenoviral vector production
3. Development of a chromatographic purification and TFF concentration process for adenoviral vectors
4. Development of a chromatographic purification and TFF concentration process for retroviral vectors
5. Development of serum-free, single cell suspension production process for adenoviral vectors
6. Actively involved in process harmonization activities with a major pharmaceutical company for adenoviral vector production
7. Successful development of a production and chromatographic purification process for adeno-associated viral vectors.
8. Involved in the development of novel liposome based non-viral gene delivery formulations
9. Have extensive experience and knowledge in process development and chromatography purification for the most commonly used gene delivery vehicles including: retroviral, adenoviral, adeno-associated viral and non-viral vectors
10. Manufacturing of retroviral, adenoviral and adeno-associated viral vectors in different organizations under GMP regulations.

TECHNOLOGY TRANSFER EXPERIENCE

Successfully managed the transfer of large-scale retroviral, adenoviral and adenoviral associated viral vector production and column purification process from Process Development Group to cGMP manufacturing for clinical production

Cost analysis of production and purification process and impact on marketable products

PROCESS VALIDATION

Successfully validated large-scale adenoviral vector production and column purification process in a cGMP environment

Work closely with QC and QA for process validation and transfer to GMP production

Design and implement process validation plans for CMC filing

cGMP EXPERIENCE

Actively involved in the preparation of CMC document for FDA submission for later stage clinical trials

Have good understanding of cGMP regulations and implementation.

Participated in the design of GMP facility and maintenance

Preparation and review of SOP and batch records

Manufacturing of clinical grade viral products for gene therapy in a GMP compliant environment

RESEARCH AND DEVELOPMENT INTEREST AREA

Cell culture and downstream processing and column purification for large-scale retroviral vector production for gene therapy

Cell culture and downstream process and column purification for large-scale adeno-associated viral vector production for gene therapy

- Bioreactor for large-scale cell culture for AAV production from cell lines in serum-free media
- Unit operation for downstream processing, tangential flow filtration
- Chromatographic purification process for AAV

Cell culture and downstream process and column purification for large-scale adenoviral vector production for gene therapy

- Large-scale bioreactor cell culture process, stirred tank for serum-free suspension culture and Cellcube system for attached cells
- Chromatographic purification process for adenoviral vectors

Formulation for viral vectors for gene therapy

- Development of lyophilization formulation and technology that can be used for lyophilization of viral vectors for gene therapy
- Development of stable, liquid formulation for adenoviral and AAV vectors

Non-viral gene delivery system

Development of stable liposome based gene delivery formulations
Efficient gene delivery observed in animal studies

High cell density bioreactor system

Design of high cell density bioreactor system for viral vector production

Process optimization and cell metabolism in high cell density animal cell cultures

Optimization of high-density animal cell culture systems with a focus on oxygenation and nutrient metabolism

AFFILIATIONS

1. European Society of Animal Cell Technology (ESACT)
2. AAAS
3. American Chemical Society
4. American Institute of Chemical Engineers
5. The American Society of Gene Therapy
6. PDA

PATENT

1. Bioreactor for retroviral vector production, **US 5,563,068**
2. Purification of retroviral vector, **US 5,661,022**
3. Method for the purification and production of adenoviral vectors, **US 6,194,191**
4. Formulation of adenoviral vectors for gene therapy, US patent applied, 60/108,606
5. Novel chromatographic purification process for AAV vectors, US patent applied

PUBLICATIONS

1. Zhang, S. Xu, D. (1989) Preparation of microcarriers for culturing animal cells. J. East China University of Chem. Technol. 15(1) 1-4
2. Zhang, S. and Ding, J. (1989) Rheological properties of filamentous mycelia fermentation broths. J East China University of Chem. Technol. 15(4) 470-477
3. Zhang, S. and Ding, J. (1989) Rheological properties of pellet mycelia fermentation broths. J. East China University of Chem. Technol. 15(4) 478-484
4. Handa-Corrigan, A., Zhang, S. and Brydges, R. (1992) Surface-active agents in animal cell culture. Animal Cell Technology. Vol5, 279-301 (eds. R.E. Spier and J.B. Griffiths). Academic Press, London
5. Handa-Corrigan, A. Chadd, M, Garcia de Castro, A. Zhang, S. (1992) A defined serum-free medium for diverse cell culture applications. Animal Cell Technology.

155-157 (eds. R.E. Spier, J.B. Griffiths and C. MacDonald), Butterworth-Heinemann, Oxford.
6. Handa-Corrigan, A., Kwasowski, P., Zhu, J., and Zhang, S. (1992) Pluronic adsorption effects in cell culture media. Animal Cell Technology. 117-121 (eds. R.E. Spier, J.B. Griffiths and C. MacDonald), Butterworth-Heinemann, Oxford.
7. Zhang, S., Handa-Corrigan, A., and Spier, R.E. (1992) Oxygen transfer properties of bubbles in animal cell culture media. Biotechnol. Bioeng. 40, 252-259
8. Zhang, S., Handa-Corrigan, A., and Spier, R.E. (1992) Foaming and media surfactant effects on the cultivation of animal cells in stirred and sparged bioreactors. J. Biotechnol. 25, 289-306
9. Zhang, S., Handa-Corrigan, A., and Spier, R.E. (1993) A comparison of oxygenation methods for high-density perfusion cultures of animal cells. Biotechnol. Bioeng. 41, 685-692
10. Zhang, S., Handa-Corrigan, A., and Spier, R.E. (1992) Kinetics of cell metabolism and antibody production in high-density perfusion cultures. Published in the proceedings of JAACT'92
11. Handa-Corrigan, A., Nikolay, S., and Zhang, S., (1994) Rationalization of the design of high-density perfusion cultures for suspended animal cells. Animal Cell Technology 1230-1236 (eds. R.E. Spier, J.B. Griffiths and C. MacDonald), Butterworth-Heinemann, Oxford.
12. H. Kotani, P. Newton, S. Zhang, Y. Chiang, G, McGarrity (1994) Improved methods for retroviral vector transduction and production for gene therapy. Human Gene Therapy 5, 19-28

CONFERENCE PRESENTATIONS

1. Oxygen transfer properties of bubbles in animal cell culture media. Zhang, S., Handa-Corrigan, A., and Spier, R.E. 4th Chemical Congress of North America, New York City, August 25-30, 1991
2. Continuous large-scale production of retroviral vectors in a novel packed-bed air-lift bioreactor. S. Zhang, P. Newton, G. McGarrity, and H. Kotani. The Williamsburg BioProcessing Conference, Nov. 8-10 1994
3. Production and downstream processing of retroviral vectors. G. McGarrity, H. Kotani, and S. Zhang. 2nd meeting of European Society of Human Genetics, London, 1994
4. Production of high titer retroviral vectors. Zhang, S., McGarrity, G., Newton, P., and Kotani, H. Keystone Symposia on Molecular and Cellular Biology, Steamboat Springs, CO, March 1995
5. An HPLC method for evaluation of the production and purification of a type 5 recombinant adenoviral vector encoding human p53. Z. Wu, D. Wilson, and S. Zhang. The Williamsburg BioProcessing Conference, Nov. 8-10, 1996
6. Production of adenoviral vectors in attached and suspension cultures. T. Cho, S. Gallagher, and S. Zhang. The Williamsburg BioProcessing Conference, Nov. 3-6, 1998

7. Development of a lyophilization process for long-term storage of adenoviral vectors. Z. Wu, S. Gallagher, D. Wilson, and S. Zhang. The Williamsburg BioProcessing Conference, Nov. 16-19, 1998
8. Process development and production of rAAV vectors for gene therapy applications using a novel infection method. S. Zhang. IBC International Symposium, Arlington VA, 1999
9. An Ad/AAV-hybrid production system for scalable and efficient AAV vector manufacture. S. Zhang, R. Peluso, S. Godwin, J. Richardson, and V. Himes. The Williamsburg BioProcessing Conference, Nov. 6-9, 2000
10. Production of adenoviral vectors for gene therapy in serum free suspension cultures. S. Zhang, H. Pham, S. J. Senesac, and S. Gallagher. The Williamsburg BioProcessing Conference, Nov. 11-14, 2002

REFERENCES

Available upon request

Fields Virology, Third Edition
edited by B.N. Fields, D.M. Knipe, P.M. Howley, et al.
Lippincott - Raven Publishers, Philadelphia © 1996

CHAPTER 67

Adenoviridae: The Viruses and Their Replication

Thomas Shenk

Adenoviruses were first isolated and characterized as distinct viral agents by two groups of investigators who were searching for the etiologic agents of acute respiratory infections. In 1953, Rowe and colleagues (389) observed the spontaneous degeneration of primary cell cultures derived from human adenoids. The pathogenic changes proved to result from the replication of previously unidentified viruses present in the adenoid tissues. In 1954, Hilleman and Werner (212) were studying an epidemic of respiratory disease in army recruits, and they isolated agents from respiratory secretions that induced cytopathic changes in cultures of human cells. The viruses discovered by the two groups were soon shown to be related (233), and they were initially called *adenoid degeneration* (AD), *respiratory illness* (RI), *adenoidal-pharyngeal-conjunctival* (APC) or *acute respiratory disease* (ARD) agents. In 1956, the agents were named *adenoviruses*, after the original source of tissue (adenoid) in which the prototype viral strain was discovered (116). Epidemiologic studies confirmed that adenoviruses were the cause of a large number of acute febrile respiratory syndromes among military recruits (1,105,151). It soon became clear, however, that adenoviruses were not the etiologic agents of the common cold; they are responsible for only a small portion of acute respiratory morbidity in the general population and about 5% to 10% of respiratory illness in children. Besides respiratory disease, adenoviruses cause epidemic conjunctivitis (242), and they have been associated with a variety of additional clinical syndromes—perhaps most notably, infantile gastroenteritis (136,510).

During the first years after their discovery, many viruses belonging to the same general group were isolated from humans, and agents with similar properties were obtained from a variety of animal species, including monkeys (234) and mice (189). Today well over 100 members of the adenovirus group have been identified which infect a wide range of mammalian and avian hosts. All of these viruses contain a linear, double-stranded DNA genome encapsidated in an icosahedral protein shell measuring 70 to 100 nm in diameter.

In 1962, Trentin and his colleagues (456) made a seminal discovery, showing that human adenovirus type 12 in-

T. Shenk: Howard Hughes Medical Institute, Department of Molecular Biology, Lewis Thomas Laboratory, Princeton University, Princeton, New Jersey 08544-1014.

duced malignant tumors following inoculation into newborn hamsters. This was the first time that a human virus was shown to sponsor oncogenesis. So far, no epidemiological evidence has been reported linking adenoviruses with malignant disease in the human; although there is one report of adenovirus-related RNA in neurogenic tumors (235), extensive searches have generally failed to find adenovirus nucleic acids in human tumors (170,295). Nevertheless, the ability to induce tumors in animals and to transform cultured cells has established adenovirus as an important model system for probing the mysteries of oncogenesis.

As the interest in adenoviruses as tumor viruses intensified, their virtues as an experimental system became evident. The prototype human adenoviruses are easily propagated to produce high titer stocks, and they initiate highly synchronous infections of established cell lines. Further, the viral genome is readily manipulated, facilitating the study of adenovirus gene functions by mutational analysis. Studies of adenovirus-infected cells have made numerous contributions to our understanding of viral and cellular gene expression and regulation, DNA replication, cell cycle control, and cellular growth regulation.

Perhaps the signal contribution to modern biology of the adenovirus system has been to host the discovery of mRNA splicing. Studies on the biogenesis of viral mRNA first demonstrated that many mRNAs were produced from a large nuclear transcript (17,480), and subsequent analysis of the structure of adenovirus mRNAs revealed the existence of introns (31,77). Today, the utility of adenovirus as a vector for gene therapy is the subject of intense exploration. This chapter will overview the structure of the adenovirus particle, the adenovirus replication cycle in human cells, its ability to oncogenically transform cells, and its interactions with host cells and host organisms. Details of adenovirus transformation are also considered in Chapter 11, and a discussion of the pathogenesis, clinical syndromes, epidemiology, techniques for diagnosis, modes of treatment, and the utility of adenoviruses as vectors will follow in Chapter 68.

CLASSIFICATION

The adenoviruses constitute the *Adenoviridae* family of viruses, which is divided into two genera, *Mastadenovirus* and *Aviadenovirus* (341). Whereas the *Aviadenovirus* genus is limited to viruses of birds, the *Mastadenovirus* genus includes human, simian, bovine, equine, porcine, ovine, canine, and opossum viruses. Although there is antigenic cross-reactivity among members within each genera due to conserved epitopes located on the hexon protein of the virion (340), there is no known antigen common to all adenoviruses.

So far, 47 human adenovirus serotypes (Table 1) have been distinguished on the basis of their resistance to neutralization by antisera to other known adenovirus serotypes. Type-specific neutralization results predominantly from antibody binding to epitopes on the virion hexon protein and the terminal knob portion of the fiber protein (340,453). The various serotypes are classified into six subgroups (Table 1) based on their ability to agglutinate red blood cells (209,211,384). The central shaft of the viral fiber protein is responsible for binding to erythrocytes, and the hemagglutination reaction of an adenovirus is inhibited by antisera specific for viruses of the same type but not by antisera to viruses of different types. A variety of additional classification schemes have been explored, including subgroupings based on oncogenicity in rodents (167), relatedness of tumor antigens (305), electrophoretic mobility of virion proteins (474), and genome homologies revealed by base composition (361), cross-hybridization (145), or digestion with restriction endonucleases (475). The various schemes produce reasonably concordant groupings (Table 1), suggesting that the widely utilized classification based on hemagglutination is a reasonable standard.

TABLE 1. *Classification schemes for human adenoviruses (mastadenovirus H)*

Subgroup		Hemagglutination groups	Serotypes	Oncogenic potential		Percentage of G–C in DNA
				Tumors in animals	Transformation in tissue culture	
A	IV	(little or no agglutination)	12,18,31	High	+	48–49
B	I	(complete agglutination of monkey erythrocytes)	3,7,11,14,16, 21,34,35	Moderate	+	50–52
C	III	(partial agglutination of rat erythrocytes)	1,2,5,6	Low or none	+	57–59
D	II	(complete agglutination of rat erythrocytes)	8,9,10,13, 15,17,19,20,22–30, 32,33,36–39,42–47	Low or none (mammary tumors)	+	57–61
E	III		4	Low or none	+	57–59
F	III		40,41	Unknown		

Modified from Baum (26).

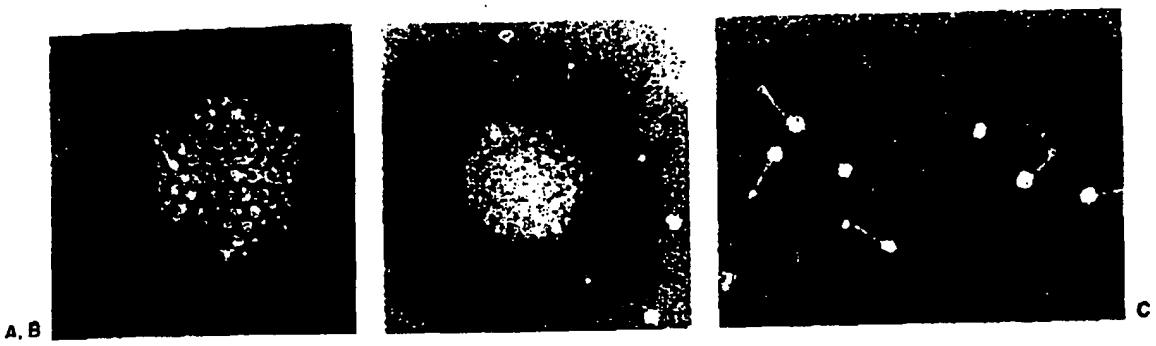

FIG. 1. Adenovirus type 5. A: The virion is an icosahedron. One of the 240 hexon capsomeres surrounded by 6 hexons and one of the 12 penton capsomeres surrounded by 5 hexons are marked (*dots*). B: Six of the twelve fibers that are present on each virus particle are shown projecting from penton capsomeres located at the verticies of the icosahedral capsid. C: Free penton capsomeres containing penton base and fiber. (×285,000.) From Valentine and Pereira (459), with permission.

VIRION STRUCTURE

Adenoviruses are icosahedral particles (20 triangular surfaces and 12 vertices) that are 70 to 100 nm in diameter (219) (Fig. 1). The particles (virions) contain DNA (13% of mass), protein (87% of mass), no membrane or lipid, and trace amounts of carbohydrate since the virion fiber protein is modified by addition of glucosamine (169,237, 238). Virions consist of a protein shell surrounding a DNA-containing core. The protein shell (capsid) is composed of 252 subunits (capsomeres), of which 240 are hexons and 12 are pentons (154). As suggested by their names, penton and hexon subunits are surrounded by five and six neighbors, respectively. Each penton contains a base, which forms part of the surface of the capsid, and a projecting fiber whose length varies among different serotypes (338,339).

Virion Polypeptides and DNA

Most of the structural studies of adenoviruses have focused on the closely related adenoviruses type 2 and 5 (Ad2 and Ad5). Electrophoretic analyses of purified virions disrupted with sodium dodecylsulfate was employed initially to identify structural polypeptides (238,296,465). Comparison of electrophoretic results with genomic open reading frames (ORFs) suggests there are probably 11 virion proteins. These proteins are numbered by convention (297), with no polypeptide I since the moiety originally designated I proved to be a mixture of aggregated smaller molecules.

The outer shell of the virion or capsid is comprised of seven known polypeptides. Polypeptide II (967 amino acids) is the most abundant virion constituent. The hexon protein is comprised of three tightly associated molecules of polypeptide II (221), and this trimeric protein is often referred to as the *hexon capsomere*. Polypeptides VI (217 amino acids), VIII (134 amino acids), and IX (139 amino acids) are associated with the hexon protein after various isolation procedures (125). All three polypeptides likely stabilize the hexon capsomere lattice, and polypeptides VI and VIII probably bridge between the capsid and core components of the virion. Five copies of polypeptide III (571 amino acids) associate to form the penton base protein (465), which is found at each vertex of the icosahedral particle. Polypeptide IIIa (566 amino acids) is associated with hexon units that surround the penton after pyridine dissociation of virions, and it probably links adjacent facets of the capsid and serves a bridging function between hexons and polypeptide VII of the core (124,125). Polypeptide IV (582 amino acids) forms the trimeric fiber protein (465) which projects from the penton base at each vertex of the icosahedron. The combination of penton base plus fiber is called the *penton capsomere*. All human adenoviruses examined to date encode a single fiber protein with the exception of Ad40 and Ad41 which encode two fiber proteins and incorporate both polypeptides into their virions (254). Since the fiber interacts with a cellular receptor protein, these viruses might recognize two independent receptors. Hexon and penton capsomeres are the major components on the surface of the virion, and their constituents, polypeptides II, III, and IV, contain tyrosine residues that are exposed on the virion surface and can be labeled by iodination of intact particles (124).

The core of the virion contains four known proteins and the viral genome. Polypeptides V (368 amino acids), VII (174 amino acids), and mu (19 amino acids) are basic, arginine-rich constituents of cores prepared by disruption of virions (223,391), and all three proteins contact the viral DNA (5,66). The function of the mu protein is unknown (223). Polypeptide VII is the major core protein, and it probably serves as a histonelike center around which the

viral DNA is wrapped (66,312). Polypeptide V can bind to a penton base (124) and it might bridge between the core and capsid, positioning one relative to the other. The fourth protein present in the core is the so-called terminal protein (671 amino acids), which is covalently attached to the 5' ends of the viral DNA. It was first identified indirectly by its ability to mediate circularization of the viral DNA through a protease-sensitive, noncovalent interaction (382). The circularizing agent was shown to be a 55-kd protein covalently attached to each 5' end of the viral DNA (378), and the protein was subsequently visualized on viral DNA by avidin-biotin labeling (45). The linkage between DNA and protein is a phosphodiester bond formed between the β-hydroxyl group of a serine residue residue 562 of terminal protein) and the 5' hydroxyl of the terminal deoxycytosine residue (100,420). The protein is not evident in electrophoretic analyses of virion proteins since it is present in only two copies per virion. The terminal protein serves as a primer for DNA replication (54,58,120,280,441), and it mediates attachment of the viral genome to the nuclear matrix (38,141,398).

Ad2 DNA was the first adenovirus genome to be completely sequenced (381), and its sequence includes a total of 35,937 bp. The sequence of Ad5 DNA was completed more recently (80), and portions of many other adenovirus genomes also have been sequenced. The Ad5 genome is about 95% identical to the Ad2 sequence, except in the fiber coding region, where a substantial portion of the differences underlying type specificity reside. Adenovirus DNA has inverted terminal repeat sequences ranging in size from about 100 to 140 bp, depending on the serotype (11,146,425,454,502). The inverted repeats enable single strands of viral DNA to circularize by base-pairing of their terminal sequences, and the resulting base-paired panhandles are thought to be important for replication of the viral DNA.

Capsid and Core Three-Dimensional Structure

X-ray crystallography, electron microscopy, and combinations of the two methods have been used to generate a fairly refined picture of the adenovirus capsid [reviewed in (430)]. The x-ray structure of the major capsid protein, the hexon, has been determined to a resolution of 2.9 Å (12,380). The hexon protein is a trimer comprised of three hexon polypeptides which are extensively interwoven. As is the case for polioviruses (216) and rhinoviruses (386), a β-structure stabilizes the association of the three subunits into a larger structure consisting of two domains: a triangular top facing the outside of the capsid and a hexagonal base with a central cavity. Group-of-nine hexons ("ninemers"), which can be isolated from each triangular face of the virion by treatment with 10% pyridine (362), provided insight to how interactions between hexons are stabilized. The structure of these ninemers was examined by subtracting an array of nine projected x-ray–determined hexons from scanning transmission electron microscopic images (142). The resulting two-dimensional difference image revealed density from a minor protein component extending along the hexon-hexon interfaces. Biochemical analysis had previously identified this component as polypeptide IX (465), a minor capsid constituent involved in stabilization of the capsid (85).

The three-dimensional structure of the complete adenovirus particle was determined to 35-Å resolution by image reconstruction from cryoelectron micrographs (429). A density map of the virion was generated from multiple images of the particle in different orientations. The reconstruction process relied on the known icosahedral symmetry of the virion to align the individual images. This work provided the first detailed visualization of the vertex proteins, including the penton base and its associated protruding fiber; it confirmed the earlier placement of protein IX; and it located minor capsid polypeptides at the edges of triangular facets, bridging hexons in adjacent facets. The three-dimensional structure of the virion has been refined by subtracting 240 copies of the crystallographic hexon from the cryoelectron microscopic image reconstruction (430). The difference map revealed more precisely the positions of several capsid proteins (Fig. 2). A less abundant stabilizing protein is located at each specialized position in the hexon assemblage. The penton complex fills the large gaps at the vertices, polypeptide IX stabilizes hexon-hexon contacts within a facet, polypeptide IIIa joins hexons of adjacent facets, and polypeptide VI anchors the ring of peripentonal hexons on the inside surface of the capsid and connects the capsid to the core.

The fiber protein which projects from each vertex of the particle is comprised of three copies of polypeptide IV (390,465). The amino-terminal 40 residues of each subunit are embedded in the penton base (101,482). A central extended shaft is comprised of repeating motifs about 15 amino acids in length, and the number of repeat units differs among adenovirus serotypes (172). The carboxy-terminal 180-residue segment of each subunit comprising the fiber protein contributes to formation of a terminal bulb (101). A three-dimensional model of the fiber shaft has been proposed (434), predicting that its constituent polypeptide chains form a left-handed, triple helical structure comprised of short β-strands interspersed with extended loops that follow the overall helical path. This model fits reasonably well with the cryoelectron microscopic results (430).

The adenovirus core is composed of the linear, double-stranded DNA and four virus-coded proteins. Unlike cellular chromatin which yields discrete mononucleosomal DNA fragments of about 146 bp upon digestion with micrococcal nuclease, the adenovirus core yields a heterogeneous population of DNA fragments ranging from about 50 to 300 bp in length (47,90,312,444,468,469), and these fragments are associated with polypeptide VII (312,468). Although the reason for the variability in DNA length is not understood, it nevertheless seems clear that adenovirus

lished that recombination occurs among adenoviruses of the same subgroup. Homologous recombination occurs with high efficiency during growth in coinfected cultured cells (156,438,496), and comparison of field strains with disparate neutralization (hexon) and hemagglutination (fiber) serology (210,211) indicates that such exchanges also occur in nature. Further, recombination between viruses of different subgroups appears to have given rise to Ad4, the sole member of subgroup E adenoviruses. Ad4 exhibits sequence similarity in its E1A (0 to 5 map units) and E2 regions (62 to 66 map units) to group B adenoviruses (258,451), and its hexon gene (51 to 61 map units) is immunologically related to group B viruses (342). However, the Ad4 fiber gene sequence (173) and immunological characteristics (340) are most similar to group C viruses. Thus, the simplest model for the origination of Ad4 posits a single recombinatorial crossover event somewhere between the E2 coding region of a group B virus and the fiber coding region of a group C virus (173).

Efficient adenovirus recombination requires that viral DNA replication occur (514,516), but the virus does not appear to encode any gene products that function specifically to facilitate recombination (121). Rather, it appears that single strands of DNA produced during the viral replication process, which can pair with each other or invade duplex DNAs, are the driving force behind recombination (137).

Although adenoviruses have never unambiguously been shown to integrate into the chromosomes of permissive host cells, integration does occur during the process of transformation. No specific motifs have been identified in the cellular DNA sequences at adenovirus integration sites, although patchy homologies between viral sequences and cellular target sites suggest that partial homologies probably influence the integration sites. Cellular proteins that mediate such recombination events have been partially purified (445).

REPLICATIVE CYCLE

Studies of the adenovirus replicative cycle have focused primarily on the closely related Ad2 and Ad5 viruses. These serotypes have been favored because they are easily grown in the laboratory, and an extensive collection of Ad2 and Ad5 mutant viruses have been developed. When other serotypes have been studied, their growth strategies have proven similar to the paradigm established for the prototypes. Most studies of adenovirus growth have been performed by infection of HeLa or KB cells at fairly high multiplicities of infection (>10 plaque-forming units per cell). High multiplicities of infection have been used so that all cells in the culture are synchronously infected, allowing the ordered series of biochemical events during the infectious cycle to be observed in a timewise fashion. HeLa and KB cells have been favored as hosts since they are easily propagated in large quantities, and the viruses grow in them rapidly and to high yield. These tumor cells support more rapid viral growth than "normal" human diploid fibroblasts where the replication cycle is substantially prolonged.

The replication cycle is divided by convention into two phases which are separated by the onset of viral DNA replication (Fig. 5). Early events are those which commence as soon as the infecting virus interacts with the host cell. These include adsorption, penetration, transcription, and translation of an early set of genes. Early viral gene products mediate viral gene expression and DNA replication, induce cell cycle progression, block apoptosis, and antagonize a variety of host antiviral measures. In HeLa cells infected at a multiplicity of 10 plaque-forming units per cell, the early phase lasts for about 5 to 6 hours, after which viral DNA replication is first detected. Concomitant with the onset of viral DNA replication, the late phase of the cycle begins with expression of a new set of "late" viral genes and assembly of progeny virions. The infectious cycle is completed after 20 to 24 hours in HeLa cells. At the end of the cycle, approximately 10^4 progeny virus particles per cell have been produced, along with the synthesis of a substantial excess of virion proteins and DNA that are not assembled into virions (168). Cells infected at high multiplicity seldom divide (220), so at the completion of the replication cycle, the DNA and protein content of the infected cell has increased about twofold.

While *early* and *late* are convenient terms for description of events that occur during the replication cycle, the functional distinction between early and late events is often blurred. For example, early genes continue to be expressed at late times after infection, and the promoter controlling expression of the major late transcription unit directs a low level of transcription early after infection. The viral genes encoding proteins IVa2 and DX begin to be expressed at an intermediate time (356) and thus form a "delayed early" category.

Adsorption and Entry

Attachment of Ad2 to cells is mediated by the fiber protein (290). The distal, carboxy-terminal domain of the fiber protein terminates in a knob that is presumed to bind to the cellular receptor (101). Soluble fiber protein and some fiber-specific antibodies can block virus attachment and infection (98,355,358,500). The identity of the cellular receptor remains a mystery, although three cellular membrane polypeptides have been captured on a penton-fiber affinity matrix and shown to inhibit Ad2 attachment to cells (206). Since the amino acid sequence of the knob region varies considerably among serotypes, it is possible that different adenovirus serotypes bind to different cellular receptor proteins. Attachment occurs efficiently at 0°C, but subsequent steps in the infectious process require energy and are inhibited at low temperatures (63).

adsorbed virus moving to endosomes within 10 minutes. About 90% of the virus within endosomes successfully moves to the cytosol with a half time of about 5 minutes. The movement to the cytosol is somehow triggered by the acidic pH of the endosome (351,405,437), and the penton base is believed to play an essential role in the process. The rapidity of the movement to the cytosol suggests that the virus escapes from the endosomal unit known as the *early endosome* prior to formation of a lysosome (310). The endosome appears to be substantially disrupted since DNA-protein complexes that enter the endosome together with virus particles—but not physically attached to virions—also escape to the cytosol with high efficiency (512). Virus particles are transported across the cytoplasm to the nucleus by a process that probably involves microtubules (94,293). *In vitro* studies have shown that virions can attach to microtubules through the hexon, and some drugs that interact with microtubules can inhibit adenovirus infection (94). About 40 minutes after penetration, virus particles can be seen at nuclear pore complexes by electron microscopy, suggesting that DNA release occurs at the nuclear membrane. After 120 minutes, about 40% of internalized particles have released their DNA free from hexon proteins, although it is not known what portion of this released DNA is localized to the nucleus.

During the internalization process, there is a sequential disassembly of the virion (166). The disassembly occurs by selective dissociation and proteolytic degradation of virion constituents. First, the proteins at the vertices of the particle are lost. Polypeptide IV, which trimerizes to form the fiber; polypeptide IIIa, located near the peripentonal hexons linking adjacent facets of the capsid; and polypeptide III, which forms the pentameric penton base, are substantially lost by 15 minutes after penetration of the cell. Polypeptides IV and IIIa dissociate somewhat more rapidly than III, and this could reflect a need to free the penton base for a role in escape from the endosome. Polypeptide VIII dissociates from the particle as the penton capsomeres are lost, and, shortly after entry of the particle to the cytosol, polypeptide VI is degraded. The internal location of polypeptide VI suggests that it might be degraded by the virus-coded, DNA-dependent protease (299,450,483). Both polypeptides VI and VIII bridge from the DNA core to the capsid, and their loss should prepare the particle for release of its DNA. Somewhat later polypeptide IX, which stabilizes hexon facets, exits from the infecting particle; finally, the DNA-containing core is freed from hexons. Thus, the virion, which is very stable outside the cell, is dismantled by an ordered elimination of structural proteins upon entry to the cell so that it can deliver its DNA to the nucleus.

The nature of the events that signal disassembly are mostly unknown. Acidification induces exit of the infecting particle from the endosome to the cytosol, but disassembly of the vertex constituents can occur in the absence of acidification (166). It seems likely that the signal for the initial dissociation of the fiber from the penton is a determining event that initiates a cascade in which each sequential disassembly event is driven by changes in the structure of the virion that result from previous events.

The components and structure of the DNA-protein complex that reach the nucleus have not been described, although it is known that protein V is removed from the core before it enters the nucleus (166). The viral DNA has been reported to convert into a structure that can be digested with a low concentration of DNase I to generate DNA fragments that form a ladder pattern similar to that of cellular chromatin upon electrophoresis (444), suggesting that cellular histone proteins might replace polypeptide VII, the major core constituent, before the infecting DNA is transcribed. However, using sensitive assays, others have been unable to detect nucleosomal particles comprised of viral DNA and cellular histones (468). It is possible that the viral genome is expressed as a chromosomal structure containing viral basic proteins rather than cellular histones.

When the viral DNA reaches the nucleus, it associates with the nuclear matrix through its terminal protein (38,141,398). DNAs from mutant Ad5 variants with lesions in their terminal protein genes fail to become tightly associated with the nuclear matrix, and they also fail to be efficiently transcribed (398). This correlation between nuclear matrix association and activation of transcription suggests the two events are functionally interrelated. Apparently, the terminal protein, which arrives with the infecting genome, is the first viral gene product that functions within the nucleus to initiate the program of viral gene expression.

Activation of Early Viral Genes

There are three main goals of early adenovirus gene expression. The first is to induce the host cell to enter the S phase of the cell cycle, providing an optimal environment for viral replication. As will be discussed later (see "Interactions with the Host" section), both E1A and E1B gene products play roles in this process. The second is to set up viral systems that protect the infected cell from various antiviral defenses of the host organism. The E3 and VA RNA genes contribute to these defenses, and these will also be discussed later (see "Virus-Host Interactions" section). The third is to synthesize viral gene products needed for viral DNA replication. All three of these goals depend on the transcriptional activation of the viral genome, and the principal activating proteins of adenovirus are encoded by the E1A gene [reviewed in (332,412)].

E1A is the first viral transcription unit to be expressed after the viral chromosome reaches the nucleus. Transcription of the E1A unit is controlled by a constitutively active promoter that includes a duplicated enhancer element (198,200). The E1A unit encodes two mRNAs during the early phase of infection (Fig. 6). Three additional E1A mRNA species accumulate later in the infectious cycle (428,458), but no definitive function has been described

domain of E1A (118,148,346). E1A can repress the action of AP1 through its consensus binding sites in the collagenase and stromelysin genes (346), and enhance the ability of AP1 to activate through the ATF sites (to which AP1 can bind) of adenovirus early genes (117,184). The AP1 transcription factor is a dimeric molecule which is generally comprised of fos and jun family members, although jun family members can also heterodimerize with members of the ATF family (30). E1A repression appears to result from a block to DNA binding by c-fos/c-jun heterodimers (177), and activation by E1A through AP1 occurs, at least in part, by an indirect mechanism. E1A proteins cooperate with cyclic AMP to induce the level of AP1, which activates transcription of viral genes (323). The E1A-mediated induction of AP1 activity results from transcriptional activation of the c-fos and junB genes through their TATA motifs (260), and the c-jun gene has been shown to be induced through an ATF-like element in its promoter, which probably responds to a c-jun/ATF-2 heterodimer (461). So, E1A can activate transcription indirectly through AP1 binding sites by inducing AP1 activity, and E1A can activate through ATF binding sites either directly by binding to ATF-2 or indirectly by inducing AP1 activity.

Expression of the adenovirus VA RNA genes is also induced by the E1A proteins (214,215,513). These genes are transcribed by RNA polymerase III, and it appears that the activity of transcription factor IIIC is altered by the E1A proteins.

Besides the E1A proteins, two additional early gene products have been shown to activate adenovirus promoters. The E4-17 kd polypeptide binds to E2F, with two molecules of the E4 protein apparently bridging between a pair of E2F proteins and causing them to bind cooperatively to the pair of E2F binding sites within the E2 promoter (183,231,328,372). The E2 DNA-binding protein can activate a variety of early adenovirus promoters as well as the major late promoter (60,318), but as yet the mechanism by which it stimulates transcription is unclear.

Individual adenovirus early promoters are activated by multiple mechanisms. For example, the E2 promoter can be activated by E1A through its TATA motif, ATF site, or E2F sites. In addition, the E4-17 kd protein contributes to activation at the E2F sites, and the E2 promoter can also be induced by the E2 DNA-binding protein. Perhaps the activation pathways function with different efficiencies within various cell types, and these apparently redundant activation mechanisms have evolved to insure that early viral promoters will be efficiently activated in a variety of different cell types as the virus spreads within its infected host.

Generally, adenovirus early genes remain active throughout the viral replication cycle, although the rate at which they are transcribed slowly declines. In part, the decline results from cell death. However, there are three known down-regulatory events, and in each case it appears that a viral protein, which accumulates in response to an activation event, subsequently acts to inhibit continued transcriptional stimulation mediated by one or more early promoter elements. First, the E1A proteins can repress the activity of a variety of known enhancers, including the enhancer residing upstream of the E1A gene itself (40,470). This down-regulatory function correlates with the ability of the E1A protein to bind to a cellular protein, p300 (383,426,478); the consequences of this protein-protein interaction will be considered further below (see "Activation of the Host Cell" section). Second, whereas the E2 DNA-binding protein activates some promoters, it appears to inhibit transcription from the E4 promoter (181,335) by an unknown mechanism. Third, the induction of AP1 activity by E1A and cyclic AMP is transient (323). It is antagonized by the E4-14 kd polypeptide which accumulates in response to the E1A-mediated activation of the E4 gene (322). Mutant viruses unable to produce the E4-14 kd protein are viable, but more cytopathic than the wild-type virus, suggesting that it is important to counterbalance the E1A-mediated induction of AP1 to preserve integrity of the host cell for the replicating virus. The E4-14 kd protein binds to protein phosphatase 2A, and phosphatase activity is essential for the down-regulatory event (260).

Once early mRNAs have been synthesized, they are translated on polysomes together with cellular mRNAs. Initially, they do not appear to enjoy any competitive advantage, but as the infection enters the late phase, cellular mRNAs are excluded from polysomes (see "Activation of Late Gene Expression" and "Host Cell Shutoff" sections).

Activation of the Host Cell

Adenovirus infection has long been known to induce quiescent cells to enter the S phase of the cell cycle, creating an environment optimally conducive to viral replication [reviewed in (454)]. Modulation of the cell cycle is primarily a function of the E1A proteins, and the modulation is mediated by their CR1, CR2, and nonconserved amino-terminal domains. The key to understanding how E1A proteins manipulate cell cycle regulation came from the observation that a set of cellular proteins can be coimmunoprecipitated with the E1A proteins (157,186,224,506). The main coprecipitating cellular polypeptides have molecular weights of about 33, 60, 80, 90, 105, 107, 130, 300, and 400 kd (Fig. 7); and several of these polypeptides have been shown to interact directly with E1A proteins (Table 2). The 105-kd moiety was the first to be identified (492). It is the retinoblastoma tumor suppressor protein pRB which, as discussed above, regulates the ability of the E2F cellular transcription factor to activate transcription. The human papillomavirus E7 protein and the large T antigens of papovaviruses also bind to pRB (97a, 111), underscoring its importance as a target of oncoproteins encoded by DNA tumor viruses. Although E2F sites are present in the adenovirus E1A and E2 promoters and contribute to their activation, neither the papovaviruses nor papillomavirus-

most commonly mutated gene in human tumors, and the loss of p53 function or its alteration by mutation can contribute to tumor progression [reviewed in (276,473.519)]. Consistent with its role in tumorigenesis, high-level expression of p53 blocks cell cycle progression at the G_1/S boundary [reviewed in (354)]. p53 is a sequence-specific DNA-binding protein that can activate transcription when it binds to p53 response elements, and it can repress a variety of genes that lack a binding site for p53 (reviewed in 519).

It appears that p53 normally functions as a component of a G_1 checkpoint that is induced by DNA damage (104,190,284). Recent work indicates that DNA damage can transiently induce p53 levels; p53, in turn, can block cell cycle progression and contribute to the activation of genes known to be induced by DNA damage. Specifically, p53 can induce expression of the WAF1 (also termed *p21*) gene (115) whose product is able to arrest cell cycle progression (187), and it can induce the GADD 45 gene which is activated by DNA damage (251). High levels of p53 can also result in apoptosis (81,292,410,511), and this will be considered below (see "Inhibition of Apoptosis" section). Thus, the current view of p53 is that it transcriptionally activates genes that prevent entry into S phase. It is not yet clear whether transcriptional repression by p53 also contributes to this process.

The Ad5 E1B-55 kd protein binds to p53 within infected cells (394), and it can block transcriptional activation by p53. Analysis of mutant E1B-55 kd proteins has revealed a strict correlation between the ability of E1B to block p53-mediated transcriptional activation and its ability to cooperate with E1A in the oncogenic transformation of cells (507). As will be discussed below (see "Oncogenesis" section), the E1A and E1B proteins of adenovirus are oncoproteins, and they cooperate to transform cultured cells. Oncogenic transformation is a manifestation of the ability of these proteins to interfere with the normal function of tumor suppressor proteins. Thus, the correlation between a block to transcriptional activation and transforming activity of the viral protein suggests that the E1B-55 kd protein antagonizes the ability of p53 to influence cell cycle progression by blocking its activation function. The E1B-55 kd protein binds to the N-terminal, acidic transcriptional activation domain of p53 (248), and this suggests that the viral protein might simply mask the activation domain. Steric hindrance might contribute to the ability of E1B-55 kd to block activation by p53, but it is not the entire mechanism. The E1B-55 kd protein can inhibit transcription if it is artificially anchored to a promoter, indicating that it can actively repress transcription (508). Apparently, the interaction of E1B-55 kd with p53 serves to bring the viral protein to the promoter, where it not only blocks the activation function of p53 but it also actively represses transcription. So, like the E1A proteins, the E1B-55 kd protein binds to a tumor suppressor protein, antagonizes its normal activity, and helps to deregulate cell cycle progression.

In contrast to the E1A proteins, expression of the E1B-55 kd protein alone is not sufficient to stimulate quiescent cells to enter the S phase of the cell cycle. This is consistent with the observation that deletion of both p53 alleles does not directly lead to the loss of regulated cell division (107). Nevertheless, p53 is targeted by a variety of tumor virus oncoproteins: SV40 large T antigen binds to p53, blocks its DNA-binding activity, and interferes with its ability to activate transcripton (25,131); and human papilloma virus type 16 E6 protein complexes with p53 and cooperates with a cellular protein to promote its degradation (399). Presumably, then, the E1B-55 kd protein collaborates with the E1A proteins to more effectively activate quiescent cells. Further, since the E1A proteins somehow stabilize p53, increasing its steady state level (291), the E1B proteins must contribute to the activation of quiescent cells by preventing the implementation of a cell cycle block by elevated levels of p53. In a similar vein, as will be discussed below (see "Inhibition of Apoptosis" section), the E1B-55 kd protein helps to prevent cells from undergoing apoptosis in response to E1A activities.

It is noteworthy that the Ad12 homologue of the Ad5 E1B-55 kd protein, the Ad12 E1B-54 kd protein, can inhibit p53-mediated transcriptional activation and cooperate with E1A to transform cells (507), even though it shows no indication of p53 binding (165,521). Also, the Ad5 E1B protein can sequester a substantial portion of the cell's p53 in a discrete cytoplasmic structure outside of the nucleus (520), while the Ad12 protein cannot (521); this has been taken as further evidence that the Ad12 protein does not bind to p53. The Ad12 E1B protein might bind weakly to p53, and the resulting complex might not be sufficiently stable to be detected by the assays that have been employed. p53 is stabilized in Ad12 transformed cells, and the stabilization is dependent on expression of the E1B-54 kd protein (462). Stabilization is often observed for mutant p53, but the p53 present in the Ad12 transformed cells is wild type. Further, while the Ad5 E1B protein inhibited transformation by *myc* plus *ras*, the Ad12 E1B protein, like mutant p53, enhanced the number of foci produced by *myc* plus *ras*. If, indeed, there is no direct interaction between the Ad12 E1B protein and p53, these observations suggest that the Ad12 protein somehow causes wild-type p53 to be modified, perhaps by association with another cellular protein, so that it behaves in some respects like mutant p53.

Inhibition of Apoptosis

As discussed above, expression of the E1A proteins in quiescent cells can efficiently induce cellular DNA synthesis and transient cell proliferation. However, E1A expression is not sufficient to induce long-term growth of primary cells. This is because in addition to inducing proliferation, E1A proteins trigger apoptosis. Apoptosis, which is also termed *programmed cell death*, is associated with

well-defined morphological changes of the nucleus and DNA fragmentation; these characteristics distinguish it from necrosis, which is cell death characterized by extensive cytoplasmic destruction that is induced by an inhospitable environment or injury [reviewed in (488,495)].

E1A-induced apoptosis involves the induction of p53 (97,291). High-level expression of p53 can block cell cycle progression [reviewed in (354)] or it can induce apoptosis (81,292,410,511). E1A proteins stabilize p53, causing it to accumulate within the nucleus (291), and both Ad5 E1B proteins can block apoptosis induced by the E1A proteins (370). The Ad5 E1B-55 kd protein probably blocks apoptosis as a result of its ability to bind to p53 and alter its function. Either the E1B-19 kd protein or the cellular Bcl-2 proto-oncoprotein can block E1A-induced apoptosis more efficiently than the larger E1B protein (370). So far, the mechanism by which p53 induces apoptosis, as well as the mechanism by which the smaller E1B protein blocks apoptosis, remain a mystery. The E1B-19 kd and Bcl-2 proteins each can block p53-mediated transcriptional repression (411). So the correlation between the ability of these proteins to block apoptosis and to alleviate p53-mediated repression raises the possibility that p53 might mediate apoptosis, at least in part, by repressing transcription.

Adenovirus mutants that fail to produce a functional E1B-19 kd protein induce extensive degradation of host cell and viral DNA, enhanced cytopathic effect, and reduced viral yield when grown in cultured cells (359,439,491). Presumably, the inhibitory effects of premature cell death on viral propagation would be even more severe in natural infections where the viral growth cycle is slower than in cultured HeLa cells. Thus, apoptosis is an example of a cellular response to infection that has the potential to inhibit viral growth and block its spread within the infected organism. However, adenoviruses have evolved to encode a gene product that effectively blocks the cellular defense.

Adenovirus is not the only virus to carry a gene that can block the onset of apoptosis. The Epstein-Barr virus LMP-1 protein induces expression of the cellular Bcl-2 protein which blocks apoptosis (205). African swine fever virus (327), herpes simplex virus (75), and baculovirus (83) have also been reported to encode proteins likely to inhibit apoptosis.

Viral DNA Replication

As E2 gene products accumulate and the infected cell enters the S phase of the cell cycle, the stage is set for viral DNA replication [reviewed in (55,195,431)]. Ad2 or Ad5 DNA replication begins about 5 hr after infection of HeLa cells at a multiplicity of 10 plaque-forming units per cell, and it continues until the host cell dies (Fig. 5).

The inverted terminal repeats of the viral chromosome serve as replication origins. *In vivo* studies support a model in which adenovirus DNA replication takes place in two stages (271) (Fig. 8). First, synthesis is initiated at either terminus of the linear DNA and proceeds in a continuous fashion to the other end of the genome. Only one of the two DNA strands serves as a template for the synthesis, so the products of the replication are a duplex consisting of a daughter and parental strand plus a displaced single strand of DNA. In the second stage of the replication process, a complement to the displaced single strand is synthesized. The single-stranded template circularizes through annealing of its self-complementary termini, and the resulting duplex "panhandle" has the same structure as the termini of the duplex viral genome. This structure allows it to be recognized by the same initiation machinery that operates in the first stage of replication, and complementary strand synthesis generates a second completed duplex consisting of one parental and one daughter strand. Adenovirus DNA was the first eukaryotic template to be replicated *in vitro* (56), and increasingly defined cell-free systems have allowed the analysis of replication in considerable detail.

Cis-acting sequences comprising the replication origins are located within the inverted terminal repeats of the viral chromosome. Three functional domains have been defined within the terminal 51bp of the repeats. Domain A consists of the first 18 bp of the viral DNA, and it comprises a minimal origin of replication. This domain is required for replication but it supports only limited replication on its own (59,269,442,460). A cellular protein, termed *ORP-A*, binds to the first 12 bp of the genome within the minimal origin (385), but this protein does not appear essential for replication. The sequence between base pairs 9 to 18 (5'-ATAATATACC-3') is conserved among different human adenovirus serotypes, and a complex of two viral protein binds here (72,320,446): the preterminal protein and the DNA polymerase. The E2-coded terminal protein is synthesized as an 80-kd polypeptide [preterminal protein (pTP)] that is active in initiation of DNA replication (54,432), and as discussed above, is found covalently attached to the 5' ends of the viral chromosome. It is subsequently processed by proteolysis during assembly of virions to generate a 55-kd fragment [terminal protein (TP)] that is covalently attached to the genome (57), but it appears that the entire protein with its single cleaved peptide bond remains associated with the genomic termini (398). The E2-coded polymerase is a 140-kd protein with biochemical properties distinct from other known DNA polymerases (120,133). It contains both 5' to 3' polymerase activity and 3' to 5' exonuclease activity that probably serves a proofreading function during polymerization (133). Preterminal protein and the polymerase form a heterodimeric complex in solution (120,279,433,446) so they would be expected to bind to the origin as a unit.

Domain B consists of base pairs 19 to 39, and domain C includes base pairs 40 to 51. These two elements are not absolutely required for adenovirus DNA replication, but they substantially enhance the efficiency of the initiation

mation of an ester bond between the β-OH of a serine residue in the preterminal protein and the α-phosphoryl group of dCMP, the first residue at the 5' end of the DNA chain (54,280). The preterminal protein-dCMP interaction requires the presence of polymerase and it is template-dependent (58,280,441), suggesting that it occurs after the preterminal protein-polymerase complex is properly positioned on the template. The 3'-OH group of the preterminal protein-dCMP complex then serves to prime synthesis of the nascent strand by the DNA polymerase.

Chain elongation requires two virus E2-coded proteins, the polymerase and single-stranded DNA-binding protein, and a cellular protein, nuclear factor II (NFII). The virus-coded DNA-binding protein is a 59-kd phosphoprotein that migrates in SDS-polyacrylamide gels with an apparent molecular weight of 72 kd (it is often referred to as the 72-kd protein). It binds tightly and cooperatively in a sequence-independent fashion to single-stranded DNA (463). The role of the DNA-binding protein in DNA replication was first revealed by analysis of an adenovirus temperature-sensitive variant carrying a mutation within the E2 gene; its ability to replicate DNA is exquisitely temperature-dependent (464). In the presence of the DNA-binding protein, which coats single-stranded replication intermediates, the polymerase is highly processive (133,285), and this is probably the basis for its requirement in chain elongation. The highly processive nature of the polymerase likely enables it to travel the entire length of the chromosome after an initiation event at the terminus. NFII copurifies with a cellular DNA topoisomerase activity (326), and mammalian topoisomerase I will substitute for it in an *in vitro* replication reaction. NFII does not significantly enhance the synthesis of nascent chains up to 9,000 nucleotides in length, so it must be needed to overcome a DNA structural problem that arises only after extensive replication.

In sum, a set of polypeptides have been identified that mediate the initiation of adenovirus DNA replication (preterminal protein, polymerase, NFI, and NFIII) and chain elongation (polymerase, DNA-binding protein, and NFII). These polypeptides, together with a template containing an adenovirus replication origin, are sufficient to reconstitute the complete viral DNA replication reaction *in vitro*. The adenovirus E4 gene also encodes one or more products required for efficient DNA replication (43,178,486), but their role in the process is probably indirect and remains obscure.

Activation of Late Gene Expression and Host Cell Shutoff

As for most DNA viruses, adenovirus late genes begin to be expressed efficiently at the onset of viral DNA replication (Fig. 5). As described earlier, the adenovirus late coding regions are organized into a single large transcription unit whose primary transcript is about 29,000 nucleotides in length (123,333). This transcript is processed by differential poly(A) site utilization and splicing to generate at least 18 distinct mRNAs (Fig. 3). These mRNAs have been grouped into five families, termed L1 to L5, based on the utilization of common poly(A) addition sites (78,333,526). Expression of this large family of late mRNAs is controlled by the major late promoter. This promoter exhibits a low level of activity early after infection, and it becomes several hundredfold more active on a per DNA molecule basis at late times (409). There appear to be at least two distinct components that contribute to activation of the major late promoter: a *cis*-acting change in the viral chromosome and induction of at least one new virus-coded *trans*-acting factor.

The time-dependent *cis*-acting modification of the adenovirus chromosome was revealed by the sequential infection of cells with two closely related adenovirus strains whose late products could be distinguished (448). Expression from the second virus to infect was initially restricted to early polypeptides, even though the first virus to reach the nucleus was actively expressing its late products. The second viral chromosome did not express late gene products until it had completed the early phase and had begun DNA replication. Thus, gene products from the first virus did not act in *trans* to initiate late expression from the second virus.

There are several possible explanations for this observation. It might be necessary to alter the constituents of the viral chromatin during the process of DNA replication to activate the major late promoter. Unreplicated viral chromatin appears to be associated with a different set of proteins than replicated chromatin (66,99). Viral DNA might remain associated with virus-coded core proteins until cellular histones bind during replication. Such an exchange could be required to activate the major late promoter. However, this proposal seems somewhat unlikely. Adenovirus DNA does not appear to be associated with histones (468), and papovaviruses exhibit a DNA replication-coupled early-to-late switch even though they package their DNA in cellular histones. Perhaps the replication process allows transcription factors to gain access to the promoter. Histones or histonelike proteins are displaced and then reassemble on DNA during replication, and this could provide an opportunity for transcription factors to compete for binding to the DNA. Alternatively, a newly arriving viral core might simply require a period of time to decondense and become available to the transcription machinery. Chromosomal domains could become accessible to the transcriptional machinery in a defined order, and the promoter responsible for most late gene expression might become accessible only after a delay. The major late promoter is active at a low level early after infection, but this might reflect early activation by a relatively minor subset of the total infecting population of viral chromosomes. It is also possible that the viral chromosome might establish a compartmentalized environment in the nucleus, and it might take a pe-

At late times, when viral mRNAs constitute about 20% of the total cytoplasmic pool (440), they are translated to the exclusion of host mRNAs (14,509). Host mRNAs are not degraded; if they are extracted from the infected cell, they can be translated with normal efficiency in a cell-free extract (447). The translational block is not dependent on the inhibition of host cell mRNA accumulation in the cytoplasm. In contrast to most host mRNAs, β-tubulin mRNA continues to accumulate in the cytoplasm, but it is not translated (316). Also, 2-aminopurine can prevent the inhibition of translation without relieving the block to accumulation of host mRNAs (229). There are several regulatory components that cooperate to facilitate selective translation of viral mRNAs late after infection.

The first regulatory component involves the cellular protein kinase R (PKR), which is activated by double-stranded RNA that accumulates within adenovirus-infected cells (301,344). After activation, it can phosphorylate eIF-2α, inactivating the initiation factor and blocking translation. Translation of host cell mRNAs is not inhibited after infection of cells deficient in PKR activity (229,343), suggesting that the kinase, and presumably inactivation of eIF-2α, are key components of the block to host cell translation. This proposal requires that viral and host translation reside in different functional compartments so that neither are inhibited by activated kinase. The adenovirus-coded VA RNAs, which will be discussed in detail below ("Viral Antagonists of α and β Interferon" section), inhibit activation of the cellular PKR kinase. These small RNAs have been shown to copurify with viral mRNAs (303) and, as a result, they might protect viral but not cellular protein synthesis, providing a functional compartmentalization.

Inactivation of eIF-4F also contributes to selective translation in adenovirus-infected cells (230). This initiation factor binds to the cap of mRNAs and facilitates scanning of the 40S ribosome from the cap to the AUG through its intrinsic helicase activity. eIF-4F is normally activated by phosphorylation, and it becomes substantially inactivated by dephosphorylation late after adenovirus infection when cellular mRNAs are not translated. The five families of mRNAs encoded by the adenovirus major late transcription unit all contain the same 200-nucleotide-long 5' noncoding region, which has been termed the *tripartite leader sequence* (31). This 5' noncoding region is important for translation of mRNAs late but not early after infection (34,289), and it substantially lacks secondary structure (106). Thus, it has been postulated (230) that tripartite leader-containing adenovirus mRNAs can continue to be translated late after infection because the 40S ribosome can scan from cap to AUG without the need for a helicase as eIF-4F activity becomes limiting. In contrast, most cellular mRNAs are no longer translated in the absence of eIF-4F because they require the helicase to permit scanning through the more extensive secondary structure at their 5' ends.

Finally, there is a selective activation of late viral protein synthesis by the 100-kd protein encoded by the L4 family of late mRNAs (196). A mutant virus expressing defective L4-100 kd protein fails to efficiently translate its late mRNAs, but it is nevertheless able to block host cell translation. The L4-100 kd protein can bind to mRNA (3), suggesting it may function at the polysome to facilitate viral translation.

Virus Assembly and Release from the Cell

The replication of viral DNA coupled with the production of large quantities of the adenovirus structural polypeptides sets the stage for virus assembly. Trimeric hexon capsomeres are rapidly assembled from monomers after their synthesis in the cytoplasm (222). Assembly of the hexon requires the participation of a second late viral protein (the L4-100 kd protein), the same protein that stimulates late viral translation. Biochemical experiments indicate that a multimeric complex of the L4-100 kd protein binds to hexon monomers (53), and genetic analyses have demonstrated that mutations in the L4-100 kd protein can block assembly of the hexon capsomere (281,347). Apparently the L4-coded protein acts as a scaffold to facilitate assembly of trimers, but the mechanism underlying the process is unknown. Penton capsomeres consisting of a pentameric penton base and trimeric fiber assemble somewhat more slowly in the cytoplasm (222). Pulse-chase experiments indicate that the penton base and fiber assemble independently, and they join to form a complete penton capsomere (222,471). After their production, hexon and penton capsomeres accumulate in the nucleus where assembly of the virion occurs.

Mutations within a variety of viral genes can interfere with the assembly process. As would be expected, alterations in structural polypeptides that comprise the hexon or penton base can prevent accumulation of mature virions as well as subassemblies such as empty capsids (92,113,392). Alterations in the polypeptide forming the fiber, polypeptide IIIa, the E2-coded DNA polymerase, or the L1-coded 52-/55-kd protein can prevent assembly of virions and lead to the accumulation of incomplete capsid-like particles (67,92,93,113,192,392). Finally, a mutant virus with a defective L3-coded protease accumulates noninfectious virionlike particles with a series of unprocessed polypeptides (479).

Studies of mutant viruses combined with analysis of the kinetics with which polypeptides are incorporated into capsids and mature virions have provided a rough outline of the adenovirus assembly process [reviewed in (357)]. Assembly appears to begin with the formation of an empty capsid (357,436) and, subsequently, a viral DNA molecule enters the capsid. The DNA-capsid recognition event is mediated by the packaging sequence, a *cis*-acting DNA element that is centered about 260 bp from the left end of the viral chromosome (163,180,197,449). Presumably one or more proteins bind at the packaging sequence and mediate the interaction between DNA and capsid, but they re-

The Adenoviruses

Edited by
HAROLD S. GINSBERG
College of Physicians and Surgeons of Columbia University
New York, New York

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CHAPTER 2

The Architecture of Adenoviruses

M. V. NERMUT

1. INTRODUCTION

Viruses are "organized associations of macromolecules," and this definition predetermines our way of dealing with the problems of virion architecture. This review, therefore, is not a pure descriptive morphology, but aims at defining and describing the basic structural elements and complexes that constitute the adenovirion, their localization in the virion, and their mutual interactions. Since several reviews on the structural proteins of adenoviruses or virus morphology have been published over the past five years, the papers quoted herein have been selected on the basis of direct applicability to the aspect under discussion; in many cases, the reader is directed to the appropriate review for other references.

Structural research can be pursued at the level of molecular structure (primary through tertiary structure of macromolecules), of macromolecules (mainly the quaternary structure, i.e., morphology of isolated macromolecules—e.g., fibers, spikes), or of macromolecular assemblies (structure of macromolecular complexes and their mutual relationships within the virion). Electron microscopy (EM) has contributed to the studies of macromolecules (hexons, fibers) and their complexes (capsid, cores) since the very beginning of the "adenovirus era," and I will try to describe the present state of our knowledge of the architecture of this highly organized virus (Fig. 1).

EM studies of adenoviruses begun more than 20 years ago (for references, see Nermut, 1980a) have shown that the virion has an icosa-

M. V. NERMUT • National Institute for Medical Research, London NW7 1AA, England

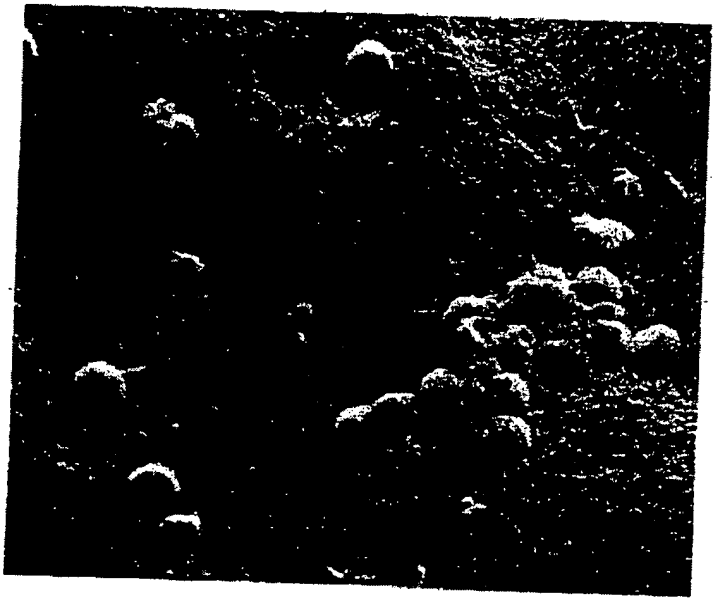

FIGURE 1. Human adenovirus invading a KB cell. Freeze-dry replica prepared 8 min post infection. × 100,000.

hedral shape and consists of two major structural complexes: the capsid, an icosahedral protein shell—and the core—an internal body that comprises the virus DNA and at least two major proteins. Before a detailed description of the individual structural elements (e.g., hexons or pentons) or complexes is presented, it is useful to recapitulate briefly what is known about the chemical composition of the adenovirus. It should be mentioned that most data used in this chapter stem from the studies of human adenovirus types 2 and 5.

II. CHEMICAL AND PHYSICAL PROPERTIES

Adenoviruses contain 11.6–13.5% of double-stranded DNA (ds DNA), the rest being protein. No lipid is present, and only about 1% is glycosylated (fiber). The molecular weight of mammalian adenovirus DNA is $20–25 \times 10^6$ (36,000 base pairs (bp)), whereas avian adenoviruses possess a DNA with a molecular weight of 30×10^6. The sedimentation constant of 31 S was reported for adenovirus type 5 (Ad 5) DNA (Harpst *et al.*, 1977) and 32 S for Ad2 DNA (Black and Center, 1979). The DNA

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III. VIRUS CAPSID

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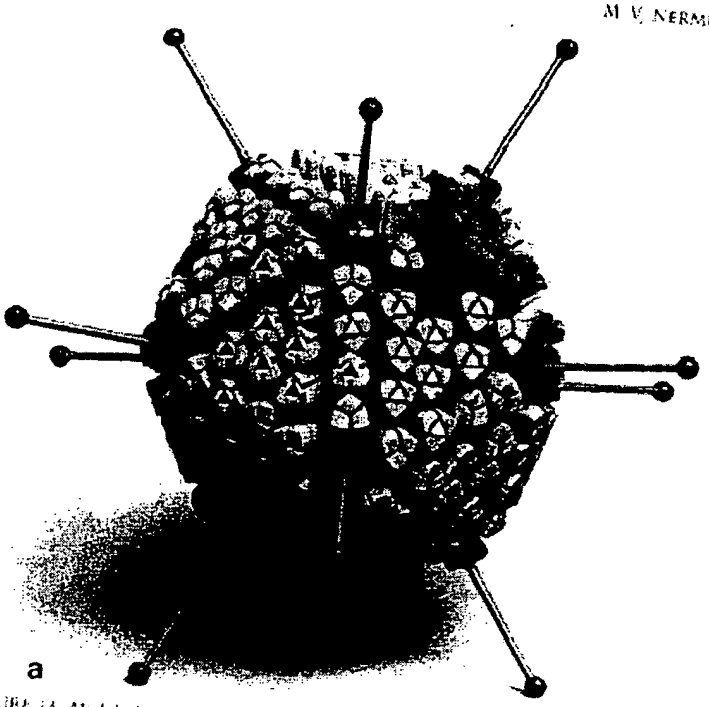

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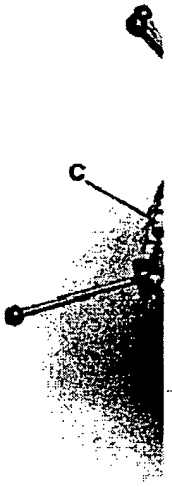

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FIGURE 13 Model of adenovirion made to scale. (a) View along the twofold axis of symmetry showing how two "groups of nine" hexons meet at the edge. In the capsid, GONs are in a left handed orientation. (b) Open view showing the "core shell" underneath the capsid and three of the superbly organized rods of the nucleocapsid proper. (c) Capsid. (CS, core shell; NP, DNA–protein complex.

Even so, this model shows the progress made over the past 20 years in our knowledge of the architecture of adenoviruses. Though the icosahedral shape and the existence of two different sorts of capsomers were recognized very early (Horne, 1962; Valentine and Pereira, 1965), the fine-structural details of the hexon and of the virus cores have been discovered only during the past five years thanks to a fruitful application of computer-assisted EM, X-ray crystallography, neutron diffraction, cryotechniques, and biochemistry.

If this interdisciplinary research continues, we shall soon know more about the molecular structure of the penton including the fiber and the function of the enigmatic VP VI. However, the most pressing problem is the organization of the DNA–protein complex. This knowledge should help us to understat
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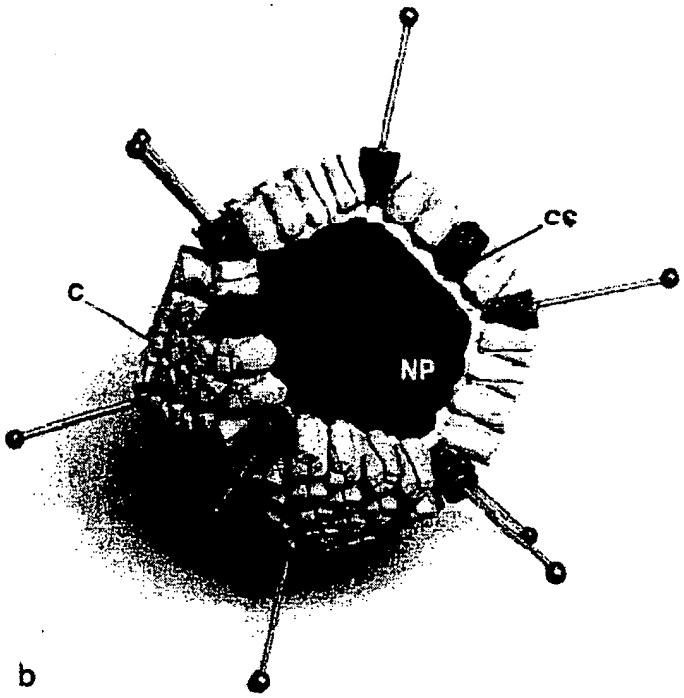

FIGURE 13 (*Continued*)

help us to understand why the core protein (VII) is possibly replaced by cellular histones before transcription and how the DNA–protein complex is assembled and packed into the icosahedral shell. Obviously, it is difficult to study assembly of a virus particle the architecture of which is not properly understood, and this is the major rationale behind the ultrastructural research.

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TABLE 1. *Viral characteristics: comparison of examples among the picornaviral genera*

	Hepatovirus	Enterovirus	Rhinovirus	Cardiovirus	Aphthovirus
In vivo					
Human serotypes	1[a]	68[a]	100	>2[b]	7
Primary hosts	Humans and other primates	Humans and other mammals	Humans and other mammals	Mice and other mammals	Cloven-footed and other mammals
Host range and tissue tropism	Narrow	Narrow to wide	Narrow	Wide	Wide
Primary habitat	Liver	GI tract	Upper resp. tract	CNS and heart	Generalized
Sensitivity[c]					
Acid[d]	Stable	Labile	Labile	Not known	Labile
pH 1	Stable	Stable	Labile	Stable	Labile
pH 3	Stable	Labile	Labile	Labile	Labile
Heat (60°C, 60 min)	Relatively resistant[e]	Sensitive	Sensitive	Resistant	Resistant
Guanidine	Resistant	Sensitive	Sensitive	Resistant	Resistant
Disoxaril[f]					
Biophysical[g]					
Buoyant density (g/cc in CsCl)	1.32–1.34	1.30–1.34	1.39–1.42	1.33–1.34	1.43–1.45
Sedimentation coefficient (S)	156–160	156–160	149	156	142–146
Mature virion proteins ($M_r \times 10^{-3}$)[a,h]					
VP1	~30.7	33.5	32.4	31.7	23.3
VP2	24.8	30.0	28.5	29.0	24.7
VP3	27.8	26.4	26.2	25.1	24.3
VP4	<2.5[i]	7.4	7.2	7.2	8.5
VPg	2.4	2.3	2.4	2.2	2.6–2.7[i]
Genome[k]					
Length (kb)	7.5	7.4	7.2	7.8–8.1	8.4
% (G+C)	38	47	40	50	43
Poly(C) tract[l]	–	–	–	+	+
Pyrimidine-rich tract[l]	+	–	–	–	–
Cross-hybridization[m]	HAVs	Enteroviruses rhinoviruses	Rhinoviruses, enteroviruses	Cardioviruses	*Aphthoviruses*

Note. **Certain data [sensitivity to guanidine, *M*, of proteins, genome length, and % (G+C)] are cited for** [illegible] strains or subtypes: HAV strain HM175/wild-type (HAV HM175), poliovirus type 1 Mahoney (PV

Poly(C) tract[l]	38	47	40	50	43
Pyr[illegible]e-rich tract[l]	–	–	–	+	+
Cru[illegible]ybridization[m]	+	–	–	–	–
	HAVs	Enteroviruses rhinoviruses	Rhinoviruses, enteroviruses	*Cardioviruses*	*Aphthoviruses*

Note: **Certain data [sensitivity to guanidine, M_r of proteins, genome length, and % (G+C)] are cited for particular strains or subtypes:** HAV strain HM175/wild-type (HAV HM175), poliovirus type 1 Mahoney (PV 1), rhinovirus type 14 (RV 14), encephalomyocarditis virus (EMCV), and foot-and-mouth disease virus type A12 (FMDV A12).

[a]See text for description of, and differences between, the seven genotypes and approximately 150 strains of HAV identified to date. Nineteen genotypes were identified for PV 1.

[b]Includes Theiler's and Vilyuisk viruses, based on analysis of their sequences, although they have previously been classified as enteroviruses.

[c]*Picornaviridae* are generally resistant to ether and are inactivated by formalin or incubation at 100∞C for 5 min.

[d]The pattern at pH 3 is similar at pH 6 except for cardioviruses, which are labile in 0.1 *M* Cl^- or Br^- at pH 3 or pH 6.

[e]Combinations of cells and virus affect sensitivity to guanidine; 2 m*M* guanidine did not interfere with HAV strain MBB replication in PLC/PRF/5 cells but did inhibit PV 1 in the same cells and HAV HM175 in BS-C-1 cells.

[f]Disoxaril, previously named WIN 51711, is a well-characterized member of a group of drugs related to arildone that inhibit viral uncoating.

[g]Primary characteristics of mature virions. In general, picornaviruses have naked capsids that are assembled in the cytoplasm and are approximately 27 nm in diameter with icosahedral symmetry.

[h]Deduced from predicted amino acid sequences.

[i]The maximum predicted M_r (2.5 kd) for HAV VP4 was deduced from the 23 amino acids encoded by the HAV HM175 open reading frame from the first AUG to the codon preceding that for the known N-terminal amino acid of VP2. Although maturation cleavage of VPO has been observed to yield VP2, VP4 had not been found in virions. In as BS-C-1 cells, translation predominantly begins at the second initiation codon, which would yield a VP4 of 21 amino acids, and there is no evidence for removal of N-terminal amino acids or myristylation as for other picornaviruses.

[j]Range of M_r for the three copies of VPg encoded by FMDV genomes.

[k]The virion RNA of picornaviruses is single-stranded, positive-sense, and polyadenylated at the 3′ terminus. Lengths shown do not include poly(A) tails.

[l]Tracts located in 5′ noncoding region (NCR): poly(C) at approximate nucleotide positions 150–275 of EMCV and 400–550 of FMDV; pyrimidine-rich at nucleotides 99–138 of HAV.

[m]Results of hybridization experiments that concurred with nucleotide sequence analysis.

GI, gastrointestinal; CNS, central nervous system.